BINUCLEATE AND TRINUCLEATE OOCYTES IN POST-OVULATION OVARIES OF RANA PIPIENS[1]

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The senior author had long been interested in the means by which diploidy can be produced in parthenogenetically developed frogs and larvae (Parmenter, 1920, 1925, 1933, 1940, 1952). He demonstrated (1952) the existence of the diploid chromosome number in three mature virgin eggs and considered this to be one source of diploidy with a known history of no delay in cleavage. He had found one instance (1940) of a diploid many-celled embryo in which, by direct observation, he knew that both polar bodies were given off and that it developed with no delayed cleavage. The discovery of a small number of binucleate ovarian eggs suggested another important possible source of such diploid parthenogenetic individuals.

In the course of undergraduate research in which nuclei were dissected from ovarian oocytes of *Rana pipiens*, several eggs were found which possessed two completely separate nuclei, and it was established that the binucleate condition was not an artifact of manipulation. The eggs used were in those stages where the nuclei had reached their fullest growth (stages 4, 5, and 6 of Duryee, 1950). Subsequently, a method was devised for scanning in ovarian tissue a large number of transparent oocytes in such early stages (stages 1 to 3 of Duryee, 1950) that the yolk would not interfere with direct observation of the cells *in situ*. It was hoped eventually that the chromosome content could be determined.

Material and Methods

Technique. The sac-like ovaries of female frogs in the post-ovulated condition were opened along their edges in Ringer solution by means of fine-pointed jeweler's forceps. The two halves, or sometimes individual pieces only, were floated onto a slide. They were scanned under low power (16 mm. objective) in the living condition, or were fixed in Bouin's fluid (which served as a stain), dehydrated, and mounted in damar. In all cases the slides were systematically surveyed and all eggs counted in which there were visible nuclei. Some cells were examined and measured first unfixed and then fixed.

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[2] All of the data herewith presented were collected and organized by Dr. Parmenter, assisted by Mr. Derezin. Unfortunately Dr. Parmenter died before the writing of the paper, which subsequently was undertaken by his wife.

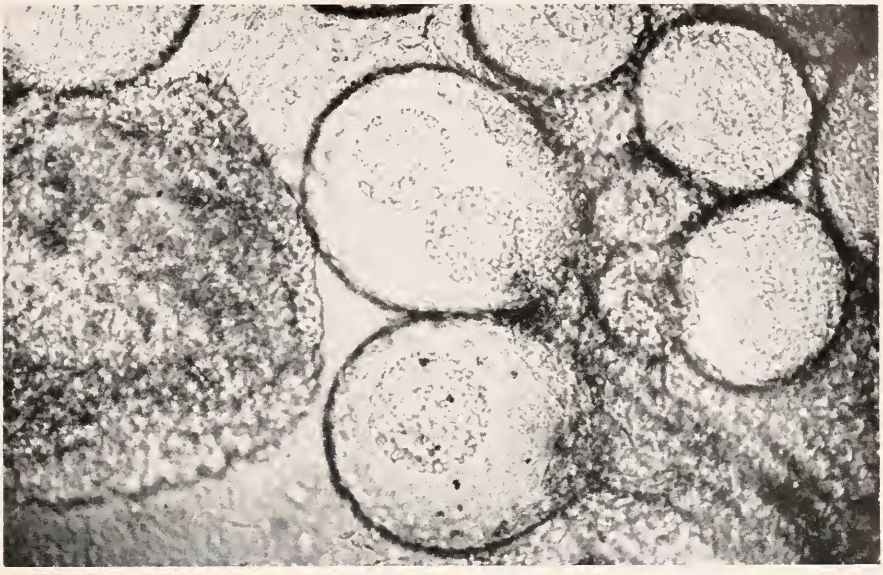

FIGURE 1. Binucleate #2 photographed *in situ* in unfixed ovarian tissue. Adjacent is the mononucleate used as a control for comparative measurements. Included also are younger oocytes and an older yolk-filled one. 430 ×

Measurements. Three different methods of measurement of egg and nucleus diameters were employed, depending on the exigencies of the moment.

Fixed eggs: When an egg with two nuclei was found, it was measured with an ocular micrometer in at least three or four diameters to correct for any variation from the spherical condition. For comparison as a "control" a single-nucleated cell of approximately the same size was located as close in the field as possible and its diameter measured. Similarly the nuclear diameters of both eggs were determined. From these data the volumes of the eggs and of their nuclei were calculated, assuming them to be spherical.

Fresh unfixed material was treated in one of two quite different ways: Usually the living binucleate was photographed *in situ*, together with a nearby mononucleate. In two cases both were fortunately in the same field. (For one, see Figure 1.) Without changing the setting of the microscope or camera, a stage micrometer was also photographed. The films were processed together and enlarged to the same magnification. Measurements were made by means of these photographs. Occasionally, when a camera was not available, camera lucida drawings were carefully made of the living binucleate, its control, and the stage micrometer, and measurements obtained from these drawings.

Extreme care was taken with all calibrations so that the measurements as finally presented in the tables are comparable with each other.

OBSERVATIONS

Fifty-six binucleate and two trinucleate eggs were found among the 249,616 small transparent primary oocytes observed. This total represented all the eggs

with visible nuclei found in 25 frogs. Thus the binucleates constituted a percentage of 0.022% or a ratio of 1 to 4,457 ova.

The percentages of multinucleates in the frogs deviated significantly from one another. Fifteen of the 25 females possessed no multinucleated eggs at all. Of the 10 with multinucleated oocytes, 8 contained very few each, only one to four. However, in the ovaries of two individuals a large number of ova with two nuclei were found, namely 14 or 0.093% in one (#44), and 24 or 0.175% in the other (#39). This indicates that certain females are more prone to produce the multinucleate condition than others where it is determined by chance. This situation, while certainly of physiological significance, is not unusual. The literature abounds with similar cases. Indeed, Parmenter's (1952) rare diploid metaphases were found in three virgin eggs, all of which came from the same female, whereas none appeared in eggs from 11 other frogs. He discussed the literature in some detail (pp. 253–254).

There was considerable variation in the number of eggs found in each frog. The range was from 5,426 to 16,066. There did not appear to be any relationship between the abundance of ova in a female and her size as indicated by the length, measured from nose tip to anus.

Three of the 25 frogs contained ovaries with considerably more eggs than the others, 13,687–16,066. The two most productive of multinucleates were among these, but a frog with 13,717 eggs possessed only one binucleate. Moreover, the first three eggs which were found with two nuclei were in an individual with small ovaries containing a total of only 5,632 eggs.

One could not predict, therefore, from external conditions such as the size of the animal, or abundance of eggs, whether multinucleate oocytes were likely to be present.

The sizes of those oocytes containing either two or three nuclei varied in diameter from very small, 0.099 mm., to 0.350 mm. (Table I). Nine ova were smaller than 0.200 mm., *i.e.*, in "stages 1 or 2" (Duryee, 1950). The rest, including the two trinucleates, were all in "stage 3." The majority of the oocytes (20) were found to measure between 0.200 and 0.300 mm. Even in one female the multinucleated eggs varied markedly in size. In female #39 which produced the 24 binucleates the diameters of the oocytes ranged from 0.163 to 0.323 mm. with most from 0.200 to 0.300 mm. Also in frog #44 (14 bi- and 1 trinucleate) the variation included the tiniest egg of all (0.099 mm.) and extended to 0.292 mm.

It is recognized that within an ovary a condition of egg growth is in progress with the various stages of growth distributed indiscriminately throughout the structure. A suggestion of this is seen in Figure 1 where one can observe a yolk-filled egg of a later stage adjacent to the mono- and binucleates which were compared, and nearby much smaller eggs. It is thus easy to see how the oocytes to be compared were chosen. The senior author was much concerned with the possibility that, due to the extended growth period of primary oocytes, the cells compared, although of the same size, might not have been growing for the same length of time. This difference in the age of the oocytes would not affect the validity of the observations, merely the conjectures as to interpretation.

In an attempt to find a clue to the chromosome content of each nucleus, the nuclear volumes of the bi- or trinucleated cells were determined and compared with that of the nucleus of the normal mononucleate which would serve as a "con-

TABLE 1

Occurrence of binucleate and trinucleate oocytes in frogs (Rana pipiens)

A. Binucleate oocytes

Binucleate oocyte number	Frog number	Nuclear volumes ($mm.^3 \times 10^{-4}$)				Cell diameters (mm.)	
		Binucleates			Mononucleate control	Binucleate	Mononucleate control
		Nucleus a	Nucleus b	Sum of a and b			
Binucleates with the volume of both nuclei approximately equal							
1	36	4.94	4.94	9.88	12.3	0.350	0.334
2	36	5.24	5.24	10.5	10.2	0.275	0.260
3	36	6.83	6.83	13.7	10.8	0.303	0.291
8	39	14.2	13.0	27.2	36.0	0.339	0.343
12	39	7.05	6.28	13.3	17.7	0.290	0.290
13	39	5.11	5.72	10.8	7.59	0.215	0.220
14	39	10.4	8.64	19.0	15.3	0.289	0.266
15	39	7.27	6.59	13.9	11.9	0.263	0.265
18	39	2.35	2.04	4.39	3.79	0.162	0.161
19	39	7.16	7.43	14.6	14.0	0.272	0.271
21	39	3.97	4.18	8.15	6.74	0.209	0.208
23	39	5.89	5.89	14.2	15.7	0.280	0.256
24	39	6.59	7.59	14.2	11.5	0.239	0.234
27	39	5.89	5.75	11.6	11.5	0.248	0.276
28	39	10.0	12.5	22.5	23.5	0.323	0.321
29	42	3.79	4.08	7.87	6.09	0.191	0.190
31	44	5.59	5.36	11.0	7.59	0.215	0.225
35	44	1.75	1.65	3.40	3.52	0.137	0.137
36	44	9.14	8.04	17.2	13.1	0.258	0.247
37	44	0.662	0.593	1.26	1.75	0.099	0.102
38	44	5.02	5.46	10.5	21.7	0.292	0.305
39	44	2.86	3.17	6.03	7.00	0.193	0.199
41	44	3.62	4.49	8.11	11.9	0.256	0.251
44	44	3.52	3.52	7.04	5.11	0.183	0.177
45	44	1.03	0.98	2.01	1.81	0.114	0.113
48	48	3.42	4.49	7.91	9.32	0.207	0.215
49	48	7.01	6.89	13.9	15.8	0.245	0.245
51	51	6.09	5.02	11.1	9.07	0.229	0.223
57	59	0.95	0.95	1.90	2.35	0.119	0.120
Binucleates with markedly unequal volumes							
30	44	1.19	0.39	1.58	1.25	0.113	0.112
43	44	8.04	4.90	12.9	8.04	0.237	0.237

B. Trinucleate oocytes

Trinucleate oocyte number	Frog number	Nuclear volumes ($mm^3 \times 10^{-4}$)					Cell diameters (mm.)	
		Trinucleates				Mononucleate control	Trinucleate	Mononucleate control
		Nucleus a	Nucleus b	Nucleus c	Sum of a, b, and c			
1	44	5.96	6.09	6.34	18.4	15.1	0.271	0.266
2	51	3.01	4.37	4.26	11.6	9.32	0.212	0.214

trol." The sum of the volumes of the twin nuclei was approximately equal to that of the single one chosen for comparison in 29 measurable cells, and markedly unequal in 2 (Table IA). Four others possessed what appeared to be unequal nuclei, but due to misshapen cells or nuclei, or visible shrinkage in one or two cases, meaningful measurements seemed impossible. These were omitted from the table, as were three ova with two nuclei of apparently the same size where the conditions were such that the measurements were questionable. The other twenty eggs, although clearly binucleate, could not properly be measured.

In none of the binucleates did the volume of either of the two nuclei approach that of the selected "control," except in the case of egg #43. It will be seen from Table I that the larger nucleus had exactly the same volume as that of the mononucleate control, and the smaller was somewhat more than half that volume. One other difference between the two nuclei of egg #43 besides size should be mentioned. There is a conspicuous dissimilarity in the granular appearance. The smaller one resembles the usual binucleate in the peripheral arrangement of the large chromatic granules (nucleoli), whereas in the larger nucleus these bodies tend to be smaller and distributed more uniformly throughout the nucleoplasm.

The total volume of the twin nuclei that were of the same size approximately equalled that of the single nucleus of the cell chosen for comparison (Table IA). There was one exception also to this statement in the case of egg #38. In this interesting cell the sum of the volumes of the two nuclei was only about one-half the volume of the nucleus of the mononucleate. Measurements of additional control cells confirmed this relationship.

While searching for the cells with two nuclei, unexpectedly the two trinucleates were found. These conformed to the general pattern for binucleates in that the diameters of the cells were in the same size range, and the sum of the volumes of the three nuclei approximated that of a "control" mononucleate. Both were located in ovaries containing cells with two nuclei also. Trinucleate #1 was found in the same lobe as binucleate #43 which possessed the large nucleus equal in size to that of the control. The three nuclei of trinucleate #1 (Table IB) were of about the same size. Since in both cases the three nuclei were at different levels within the cell a photograph was not feasible. In trinucleate #2 two of the nuclei were larger in size than the other, and equal in volume to each other. Interestingly, a similar difference in granular appearance existed between the two large nuclei and the smallest one as was described for binucleate #43. The small nucleus had fewer and larger chromatic granules whereas the two larger nuclei possessed smaller and more numerous ones.

Discussion

The following ideas were found among Doctor Parmenter's notes. No further discussion will be attempted.

The unequal-sized binucleates and the trinucleates suggest that a possible origin may have been from separated groups of chromosome vesicles. But such an origin does not seem probable for the large proportion of binucleated oocytes, the nuclei of which were of equal volume. More likely this condition arose by a nuclear division followed by a failure of cytosomic division. These binucleated cells may have been produced during the last oogonial division.

Should further information demonstrate that in some cases each of the two equal-sized nuclei contains the full set of 13 tetrads, both the normal behavior and the possible failure of one of these nuclei to give off its polar body in either meiotic division would produce various chromosome numbers in mature eggs and in embryos resulting either from parthenogenetic stimulation or from fertilization.

Literature

Information in the literature concerning multinucleate oocytes is sparse. Indeed Humphries (1956) in discussing the origin of spontaneous polyploidy in *Triturus viridescens* hesitated to "assume" (p. 120) the existence of a binucleate oocyte as a source of diploidy in such embryos. He preferred the explanation of (p. 120) "direct pathways actually seen to exist," one of which he describes in his paper on the effects of heat shock on the first meiotic division. We have now shown that binucleate primary oocytes actually do exist, at least in *Rana pipiens*. Beatty (1957) who reviewed the literature on polynuclear ovarian eggs, states that such are (p. 81) "rare but widely spread." He mentions twelve species of mammals (including man), reptiles, birds, insects, but no amphibians. Sentein (1958) was able to produce multinucleated eggs of *Triturus* and *Pleurodeles* by treatment with phenol. He saw cytoplasmic division inhibited. This treatment constitutes, of course, an unnatural source of the polynucleate condition.

In embryonic tissue Parmenter (1937, 1940) reported multinucleated erythrocytes in parthenogenetic frog larvae, also five epithelial cells each with two nuclei plus one with three. Moore (1957) has presented evidence that chromosomal vesicles constitute a basis for the origin of what she refers to as "double nuclei" (p. 209) in early embryos of diploid frog hybrids. She reviewed the literature extensively. Of interest here is her discussion of the distribution of peripheral coarse vs. diffuse fine chromatin in some of the cell nuclei of her material. She states that similar conditions were also found by King and Briggs, by Brachet, and by others. She wonders if some of the (p. 222) "so-called nuclear anomalies" found in hybrids are not really of (p. 222) "normal occurrence in the development of amphibian eggs."

The occurrence of the bi- and multinucleate condition *normally* in liver tissue of mammals including human is well known and has been reviewed recently by Inamdar (1958). By microspectrophotometric measurements of DNA in resting nuclei of mouse liver, he was able to confirm the conclusions of Beams and King (1942) and others that the origin of the binucleates is best explained by division of the nucleus without division of the cell.

No discussion will be undertaken on the often-noted polynucleate condition in tissue cultures, or in pathological material.

Summary

1. Fifty-six binucleated and two trinucleated cells were found among 249,616 young transparent primary oocytes in post-ovulation ovaries of 25 females of *Rana pipiens* (0.022%).

2. Multinucleated oocytes were absent in 15 females, present in 10. Eight of these 10 produced only one to four binucleates each; one female was the source

of 1 tri- and 14 binucleates; and another gave the remarkable number of 24 (0.175%), all binucleates.

3. The multiple nucleated condition did not seem to be correlated in any way with the size of the female, the abundance of her eggs, nor the size of the egg.

4. In none of the binucleates did the volume of either of the two nuclei approach that of the nucleus of a mononucleate of the same size. The one exception was egg #43 where one nucleus did have exactly the volume of the control, the other about half that.

In 29 of the 31 measurable binucleates the two nuclei were of approximately the same size and the sum of the two volumes equalled that of the mononucleate, except in one case where it was half.

The origin of binucleate oocytes remains uncertain; it may be connected with a final division of an oogonial nucleus that was not followed by cell division. In this case the two nuclei would both be diploid.

5. The two trinucleates conformed in general to the same pattern as the binucleates as to their distribution, size of oocyte, and the volumes of their nuclei. The sum of the three nuclear volumes approximated that of the mononucleate.

6. In two cases where the nuclei were markedly unequal in size, there was a definite difference in the appearance of their chromatic granules. These bodies were more abundant and finer in the larger nuclei, peripheral, larger and more distinct in the smaller nucleus.

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